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Regulation of insulin receptor substrate-1 by mTORC2 (mammalian target of rapamycin complex 2)

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Abstract

mTOR (mammalian target of rapamycin) responds to the presence of nutrients, energy and growth factors to link cellular metabolism, growth and proliferation. The rapamycin-sensitive mTORC (mTOR complex) 1 activates the translational regulator S6K (S6 kinase), leading to increased protein synthesis in the presence of nutrients. On the other hand, the rapamycin-insensitive mTORC2 responds to the presence of growth factors such as insulin by phosphorylating Akt to promote its maturation and allosteric activation. We recently found that mTORC2 can also regulate insulin signalling at the level of IRS-1 (insulin receptor substrate-1). Whereas mTORC1 promotes IRS-1 serine phosphorylation that is linked to IRS-1 down-regulation, we uncovered that mTORC2 mediates its degradation. In mTORC2-disrupted cells, inactive IRS-1 accumulated despite undergoing phosphorylation at the mTORC1-mediated serine sites. Defective IRS-1 degradation was due to attenuated expression of the CUL7 (Cullin 7) ubiquitin ligase substrate-targeting subunit Fbw8. mTORC2 and Fbw8 co-localize at the membrane where mTORC2 phosphorylates Ser⁸⁶ to stabilize Fbw8 and promotes its cytosolic localization upon insulin stimulation. Under conditions of chronic insulin exposure, inactive serine-phosphorylated IRS-1 and Fbw8 co-localize to the cytosol where the former becomes ubiquitinated via CUL7/Fbw8. Thus mTORC2 negatively feeds back to IRS-1 via control of Fbw8 stability and localization. Our findings reveal that, in addition to persistent mTORC1 signalling, increased mTORC2 signals can promote insulin resistance due to mTORC2-mediated degradation of IRS-1.

Keywords

insulin receptor substrate (IRS)-1 degradation; insulin resistance; mammalian target of rapamycin complex 2 (mTORC2); rapamycin; rapamycin-insensitive companion of mammalian target of rapamycin (rictor); stress-activated protein kinase-interaction protein 1 (SIN1)

Introduction

mTOR (mammalian target of rapamycin), the catalytic subunit of two evolutionarily conserved protein complexes, mTORC (mTOR complex) 1 and mTORC2, links cellular metabolism with growth and proliferation. Numerous studies have elucidated how mTORC1, composed of mTOR, raptor (regulatory-associated protein of mTOR) and mLST8

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(mammalian lethal with sec-13 protein 8), is involved in cellular metabolism [1,2]. In response to amino acids, this complex augments anabolic processes such as protein synthesis that are required for cell growth. The best-characterized function of mTORC1 in protein synthesis is phosphorylation of translation regulators, such as the 4E-BPs [eIF4E (eukaryotic initiation factor 4E)-binding proteins] and the ribosomal protein S6Ks (S6 kinases). Deregulation of mTORC1 signalling is linked to metabolic disorders such as diabetes and cancer.

mTORC2 also plays a role in metabolism, but the mechanisms are less well understood in comparison with mTORC1 [3]. This complex responds to the presence of growth factors such as insulin, but how these signals activate mTORC2 remain to be elucidated. mTORC2, composed of mTOR, rictor (rapamycin-insensitive companion of mTOR), SIN1 (stress-activated protein kinase-interaction protein 1) and mLST8, is not directly sensitive to rapamycin, but its assembly could be inhibited after prolonged exposure to rapamycin [4]. mTORC2 is linked to cellular metabolism via its regulation of the serine/threonine kinase Akt [also known as PKB (protein kinase B)]. Akt mediates the metabolic actions of insulin, which include augmenting glucose transport and promoting mTORC1 signalling to drive protein synthesis and cell growth [5]. mTORC2 controls Akt allosteric activation via post-translational phosphorylation at the hydrophobic motif residue Ser⁴⁷³, and its maturational stability by co-translational phosphorylation at the turn motif site Thr⁴⁵⁰ [6,7]. Genetic studies on mice, wherein mTORC2 is disrupted in insulin-responsive tissues, underscore the role of mTORC2 in insulin signalling. In adipose-specific *rictor*-knockout mice, fat cells have impaired insulin-stimulated Akt phosphorylation resulting in decreased GLUT4 (glucose transporter 4) translocation to the plasma membrane and diminished glucose uptake [8,9]. Muscle-specific *rictor*-knockout mice also display reduced Akt phosphorylation and insulin-stimulated glucose uptake [10]. Liver-specific *rictor*-knockout mice have hepatic loss of Akt Ser⁴⁷³ phosphorylation, constitutive gluconeogenesis, as well as impaired glycolysis and lipogenesis [11]. Recently, rapamycin treatment was shown to prolong lifespan in mice while also causing insulin resistance owing to mTORC2 inhibition [12]. These intriguing findings raise the possibility that attenuating mTORC2 signals could be beneficial for lifespan extension. Together, the above studies support a role for mTORC2 in insulin sensitivity and metabolic homeostasis.

mTOR and cellular insulin signalling

Insulin signalling is triggered by activation of the IR (insulin receptor), which then binds the IRS (IR substrate) adaptor protein. There are six members of the IRS family (IRS-1 to IRS-6), of which IRS-1 is the most well characterized. IRS-1 protein expression is markedly reduced in insulin-resistant states [13,14], although the precise mechanisms involving down-regulation remain obscure. Following insulin stimulation, IRS-1 becomes phosphorylated at tyrosine residues by the activated IR, which creates docking sites for multiple SH2 (Src homology 2)-containing proteins including the p85 regulatory subunit of PI3K (phosphoinositide 3-kinase) [15]. Whereas tyrosine-phosphorylated IRS-1 positively regulates insulin signalling, hyper-serine/threonine phosphorylation serves as a negative feedback to down-regulate IRS-1 function [16]. The majority of serine/threonine phosphorylation down-regulates IRS-1 by promoting dissociation of IRS-1 from the

receptor, blockage of specific tyrosine phosphorylation sites, cellular compartmentalization and degradation of IRS-1 [17–20]. Understanding how prolonged insulin stimulation can prompt IRS-1 down-regulation and consequent degradation would provide insights into mechanisms that lead to insulin resistance and Type 2 diabetes.

Numerous protein kinases including mTOR and members of the AGC-type kinases [protein kinase A/protein kinase G/PKC (protein kinase C)-family kinases] have been shown to mediate IRS-1 serine/threonine phosphorylation [21]. Enhanced mTORC1 signalling elevates IRS-1 serine phosphorylation, which desensitizes cells to further insulin stimulation [22,23] (Figure 1). Chronic elevation of mTORC1 signals could therefore lead to insulin resistance. Indeed, elevated phosphorylation of the sites regulated by mTORC1 and S6K has been observed in mouse models of obesity and insulin resistance [24]. Under normal conditions, feedback regulation from mTORC1 to IRS-1 can serve as a brake to attenuate growth signals and prevent uncontrolled cell growth [25]. However, this feedback loop can also enhance PI3K/Akt signalling in some cancer cells treated with rapamycin, thus exposing a possible undesirable effect of rapamycin as an anticancer therapy [26]. Thus it would be worthwhile to define how IRS-1 signals are precisely regulated by mTOR.

In addition to S6K, other members of the AGC family of protein kinases such as Akt and PKC, which are known mTORC2 targets, also phosphorylate IRS-1 at various serine/threonine sites. Site-specific IRS-1 phosphorylation by Akt has been reported to both positively and negatively regulate insulin signalling. For instance, following insulin stimulation and Akt activation, human IRS-1 is directly phosphorylated by Akt at Ser⁶²⁹ [27]. Increased phosphorylation at Ser⁶²⁹ results in a decrease of inhibitory Ser⁶³⁶ (mTORC1-mediated) phosphorylation, most likely through steric hindrance, thereby enhancing and sustaining insulin signalling. Other work suggests that Akt phosphorylates IRS-1 at Ser⁵²² to suppress insulin signalling [28]. A point mutation of Ser⁵²² to alanine increased insulin-stimulated IRS-1 tyrosine phosphorylation and downstream signalling, whereas its phosphomimetic mutation led to decreased signalling. In diabetic fat tissue, cPKC (conventional PKC) β II has elevated expression and kinase activity and plays a role in the phosphorylation of IRS-1 at Ser³³⁶ [29]. Knockout of another cPKC, cPKC α , led to enhanced insulin signalling in skeletal muscles and adipocytes [30]. Since previous studies revealed that mTORC2 plays a central role in Akt and PKC stability and allosteric activation [7,31,32], we sought to examine how IRS-1 is affected upon mTORC2 disruption.

IRS-1 signalling is decreased despite increased IRS-1 protein levels upon mTORC2 disruption

In SIN1-deficient MEFs (murine embryonic fibroblasts) wherein mTORC2 assembly is disrupted [33], IRS-1 protein expression is increased approximately 4-fold [34]. Silencing of mTORC2 components using siRNA (small interfering RNA) also enhanced IRS-1 levels. Consistent with previous findings [35], inhibition of mTORC1 by *raptor* knockdown or short-term rapamycin treatment slightly enhanced IRS-1 expression. Upon inhibition of both mTORCs via Torin1 or prolonged rapamycin treatment, IRS-1 levels became augmented. Although a previous study failed to detect changes in IRS-1 levels upon *ricor* knockdown [36], other reports have shown elevated IRS levels following prolonged rapamycin

treatment, a condition that inhibits mTORC2 [37,38]. The increased IRS levels in the latter studies were attributed to inhibition of mTORC1, which relieves the negative feedback to IRS-1. Intriguingly, despite conditions wherein mTORC2 is disrupted, Akt Ser⁴⁷³ phosphorylation could still be observed. Thus whether enhancement of IRS-1 levels under mTOR-inhibited conditions increases IRS-1 signalling capacity deserves further scrutiny.

We found that the elevated IRS-1, following mTORC2 disruption, was not due to increased transcription or translation, but due instead to defective turnover. When *de novo* IRS-1 synthesis was blocked using cycloheximide, the half-life of existing IRS-1 was approximately 4-fold longer in mTORC2-disrupted cells as compared with WT (wild-type). Pulse-chase analysis of metabolically labelled newly synthesized IRS-1 further confirmed the prolonged half-life of IRS-1. Several mechanisms for IRS-1 turnover have been reported including Ub (ubiquitin)-dependent degradation by the proteasome [39–42]. When the proteasome was inhibited using MG-132 (the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-leucinal), ubiquitylated IRS-1 accumulated in WT MEFs, but not in SIN1-deficient MEFs. Thus impaired ubiquitylation could prevent IRS-1 turnover, which might explain the increased levels of IRS-1 in mTORC2-disrupted cells.

Inhibition of IRS-1 degradation by rapamycin treatment has been shown to prolong insulin signalling [42]. Thus we examined whether the increased IRS-1 levels correlated with enhanced IRS-1 signalling despite defective Akt hydrophobic motif phosphorylation in mTORC2-disrupted cells. Surprisingly, IRS-1 phosphorylation at Tyr⁹³⁵ was dramatically diminished in SIN1-deficient MEFs. Normally, phosphorylation of IRS-1 at Tyr⁹³⁵ promotes association with PI3K, and subsequent increase of PIP3 (phosphatidylinositol 3,4,5-trisphosphate) production [15]. We therefore examined binding of the catalytic (p110) and regulatory (p85) PI3K subunits to IRS-1. Despite increased levels of immunoprecipitated IRS-1, p110 binding was not enhanced in SIN1-deficient cells, although bound and total p85 levels were elevated. Since inactive monomeric p85 can compete with the catalytically active p85–p110 heterodimer for IRS-1 binding [15,43], our results suggest that there could be attenuated PI3K activity in SIN1-deficient cells [34]. Indeed, PIP3 production following insulin re-stimulation of starved cells was diminished in *Sin1*^{−/−} MEFs. These findings support the hypothesis that, although IRS-1 levels are elevated in mTORC2-disrupted MEFs, IRS-1 signalling capacity is reduced.

Prolonged insulin stimulation increases IRS-1 serine/threonine phosphorylation, which attenuates IRS-1 signalling and precedes IRS-1 degradation [42]. We therefore analysed IRS-1 serine phosphorylation, in particular at sites regulated by mTORC1/S6K. Phosphorylation of Ser⁶³²/Ser⁶³⁵ and Ser³⁰² was induced in both WT and *Sin1*^{−/−} MEFs upon serum re-stimulation of starved cells, with levels in *Sin1*^{−/−} MEFs being proportional to the increased IRS-1 protein levels. After extended insulin stimulation, IRS-1 levels were markedly diminished in WT MEFs, whereas levels remained unchanged in *Sin1*^{−/−} MEFs. Strikingly, despite decreased IRS-1 serine phosphorylation after rapamycin treatment, tyrosine phosphorylation was not sustained in *Sin1*^{−/−} MEFs, further supporting that IRS-1 in *Sin1*^{−/−} MEFs has reduced signalling capacity. Thus our findings indicate that mTORC1/S6K-mediated IRS-1 serine phosphorylation corresponds to decreased IRS-1 signalling [34], but can be uncoupled from its degradation.

Fbw8-mediated degradation of IRS-1 is controlled by mTORC2

Following chronic insulin exposure, IRS-1 degradation occurs in a PI3K-dependent manner and involves the Ub-proteasome pathway [39,42,44–46]. Both PI3K and 26S proteasome inhibitors prevent the degradation of IRS-1, but the precise mechanism has been elusive. Whether the 26S proteasome pathway is controlled via PI3K is also unclear. In a previous study, the CUL7 (Cullin 7) E3 Ub ligase complex was demonstrated to target IRS-1 in an mTOR/S6K-dependent manner [47]. Fbw8 (also called Fbxw8), the substrate-targeting subunit of this complex, interacts with IRS-1 [47]. Deregulation of CUL7 and Fbw8 has been previously linked to growth retardation and developmental defects [48]. Mutation of several mTOR/S6K-targeted sites within IRS-1 resulted in partial resistance to CUL7/Fbw8-dependent degradation [47]. We therefore examined whether the CUL7 E3 Ub ligase complex that targets IRS-1 is responsible for accumulation of inactive IRS-1 in *Sin1*^{-/-} MEFs. Interestingly, protein expression of Fbw8, but not CUL7, was attenuated upon mTORC2 disruption or inhibition. Knockdown of mTORC2 components in adipocytes and muscle cells also led to a reduction of Fbw8 levels, which was accompanied by elevated IRS-1 expression. Fbw8 overexpression in *Sin1*^{-/-} was sufficient to reduce IRS-1 levels in a dose-dependent manner. Moreover, IRS-1 levels were increased in *Fbw8*^{-/-} MEFs similar to *Sin1*^{-/-} and *Cul7*^{-/-} MEFs [47]. Together, these results suggest that attenuated Fbw8 levels could account for defective IRS-1 degradation and, importantly, that Fbw8 itself may be regulated by mTORC2.

mTORC2 has been demonstrated previously to control protein stability of Akt and cPKC via phosphorylation at the conserved turn motif containing the sequence Ser/Thr-Pro-Pro. Fbw8 harbours a similar motif (Ser⁸⁶-Pro-Pro). Furthermore, Fbw8 was identified as a putative mTOR target in a phosphoproteomic screen [37,49]. We therefore examined whether mTORC2 can regulate Fbw8 stability via phosphorylation. The phosphate-binding tag SDS/PAGE mobility of Fbw8 from Torin1-treated cells was faster relative to untreated or rapamycin-treated cells, suggesting that Fbw8 is dephosphorylated upon mTORC2 inhibition. The dephosphorylation also coincided with reduced levels of Fbw8, consistent with Fbw8 stability being regulated by mTORC2-dependent phosphorylation. We next investigated whether mTOR could phosphorylate Fbw8 both *in vivo* and *in vitro*. Using a phospho-antibody against Ser⁸⁶, phosphorylation of Fbw8 was detected in WT, but not in *Sin1*^{-/-} MEFs. This phosphorylation was not augmented by insulin stimulation, suggesting that constitutive phosphorylation by mTORC2 is most likely to be in a co-translational manner [7]. An *in vitro* kinase assay where immunoprecipitated mTOR or rictor was used as the kinase source revealed that Fbw8 WT, but not a S86A mutant, can be robustly phosphorylated by mTORC2. This phosphorylation was barely detectable when mTOR or rictor was immunoprecipitated from Torin1-treated cells, further verifying that mTORC2 can phosphorylate Fbw8 at Ser⁸⁶. To further analyse how Ser⁸⁶ phosphorylation plays a role in Fbw8 stability, we reconstituted Fbw8-deficient MEFs with Fbw8 WT or mutant Fbw8-S86A. Upon prolonged insulin stimulation of these cells, IRS-1 protein levels became attenuated in cells expressing Fbw8 WT, whereas IRS-1 remained elevated in cells expressing Fbw8-S86A due to diminishing Fbw8-S86A levels. Our findings indicate that sufficient Fbw8 levels are necessary for proper IRS-1 turnover following insulin stimulation

[34]. They also support the hypothesis that mTORC2-mediated Fbw8 phosphorylation, which imparts stability, is critical for the IRS-1 degradation process.

The cellular compartment where IRS-1 becomes down-regulated and degraded has remained obscure. Tyrosine-phosphorylated IRS-1 associates with intracellular membrane fractions [50]. In *Tsc2*^{-/-} (tuberous sclerosis complex 2) MEFs wherein mTORC1 is hyperactive and IRS-1 is presumably hyper-serine/threonine-phosphorylated at the mTORC1-mediated sites, IRS-1 was depleted from the high-speed pellet membrane fractions [20]. These findings imply that IRS-1 could localize to the cytosol upon inactivation and thus undergo degradation in this compartment. We found that IRS-1 from WT MEFs localized predominantly with membrane-containing fractions. However, the majority of IRS-1 from *Sin1*^{-/-} MEFs was sequestered in the cytosolic fraction, further supporting the hypothesis that accumulated IRS-1 from these cells is inactive. Interestingly, Fbw8 from both WT and *Sin1*^{-/-} MEFs localized in the membrane fractions, however, only Fbw8 from WT cells translocated to the cytosol upon insulin stimulation. The increased cytosolic translocation correlated with decreasing IRS-1 levels in this compartment, suggesting Fbw8-mediated degradation of IRS-1. Consistent with this observation, Fbw8 from *Sin1*^{-/-} cells was retained predominantly in the membrane fraction, whereas IRS-1 in the cytosolic fraction remained abundant. Furthermore, the Fbw8-S86A mutant became excluded from the cytosol even after prolonged insulin stimulation, correlating with IRS-1 accumulation in this compartment. Together, these results indicate that mTORC2-mediated phosphorylation stabilizes Fbw8 and is required for insulin-induced translocation of Fbw8 to the cytosol where it targets IRS-1 for degradation (Figure 1).

Conclusion

Our findings reveal that mTORC2 is important for controlling cellular insulin sensitivity via its novel role in feedback regulation of IRS-1 levels. Under normal conditions, this mTORC2 function would curb insulin signalling and thus limit cell growth. Under pathological conditions such as insulin-resistant states, inhibiting mTORC2 could prevent IRS-1 degradation and potentially enhance IRS-1 signalling. However, mTORC2 has other important functions in insulin signalling, most notably Akt activation. Our results also revealed that chronic mTORC2 disruption/inhibition alters other signals along this pathway such as attenuated PIP3 production, IR expression and enhanced p85 levels [34]. Thus it would be crucial to identify specific mTORC2 effectors that can specifically prolong IRS-1 signals with minimal negative effects on other mTORC2 cellular functions. Fbw8, as we have discussed in the present review, is an mTORC2 substrate that can potentially be targeted to prolong IRS-1 signals.

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Abbreviations used

AGC-type kinases	protein kinase A/protein kinase G/protein kinase C-family kinases
cPKC	conventional PKC
CUL7	Cullin 7
IR	insulin receptor
IRS	insulin receptor substrate
MEF	murine embryonic fibroblast
mLST8	mammalian lethal with sec-13 protein 8
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
PI3K	phosphoinositide 3-kinase
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
raptor	regulatory-associated protein of mTOR
rictor	rapamycin-insensitive companion of mTOR
SIN1	stress-activated protein kinase-interaction protein 1
S6K	S6 kinase
Ub	ubiquitin
WT	wild-type

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Figure 1. Model of feedback regulation of IRS-1 by mTORC1 and mTORC2

Insulin signals are propagated by recruitment of IRS-1 to the IR, leading to increased mTOR (mTORC1 and mTORC2) signalling and cell growth (grey arrows). Enhanced mTORC1 signals and chronic insulin stimulation down-regulate insulin/IRS-1 signals via mTORC-mediated feedback loops (bold arrows). mTORC1 enhances serine/threonine phosphorylation of IRS-1, which excludes IRS-1 from the membrane. A feedback loop from mTORC2 also occurs wherein mTORC2 regulates stability and insulin-induced localization of Fbw8 to the cytosol. In this compartment, the Fbw8/CUL7 Ub ligase complex promotes IRS-1 ubiquitylation and degradation, which down-regulates IRS-1 signalling and cell growth. rapa, rapamycin.